13th National Congress of Parasitology

February 24 - 26, 1999

" RELEVANCE OF BIOTECHNOLOGICAL TOOLS IN THE CONTROL OF VECTORS"

February 25, 1999

WORKSHOP MANUAL

BANGALORE UNIVERSITY
&
THE INDIAN SOCIETY FOR PARASITOLOGY

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ON

"RELEVANCE OF BIOTECHNOLOGICAL TOOLS IN THE CONTROL OF VECTORS"

February 25, 1999

WORKSHOP MANUAL

Edited by :

N. J. SHETTY, JAYAPRAKASH AND K. SRIHARI

Bangalore University

&

The Indian Society for Parasitology

Organised by :

Centre for Applied Genetics

Bangalore University, Bangalore - 560 056

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PREFACE

The "13th National Congress of Parasitology" is being organised under the auspices of the Centre for Applied Genetics, Bangalore University from February 24 - 26th, 1999 at Yavanika Auditorium, State Youth Centre, Nrupathunga Road. Further, a day's workshop on "Relevance of Biotechnological Tools in the Control of Vectors" is also being organised on 25th February, 1999. I welcome all the participants who are attending the above said workshop.

In order to combat vector borne diseases, either the parasites or the vectors should be controlled. It is generally believed that vector control is more appropriate. The control of vectors faces two important challenges, namely development of resistance to insecticides and pesticides and the environmental pollution caused due to constant application of the latter. Therefore, alternate strategies such as genetic control or autocidal control, which do not involve resistance among the vectors, seems more desirable.

The Centre for Applied Genetics, has been actively carrying out research on various aspects of mosquitoes, including formal genetics, cytogenetics, genetic basis of insecticide resistance, genetic control of mosquitoes through genetic sexing strains, synthesis of transgenic species for population replacement etc.

The Centre currently offers two year M.Sc. course in Applied Genetics, which gives special emphasis for parasitic borne diseases, especially tropical diseases. With this background, it is proposed to have a day's workshop, highlighting the biotechnological methods available in the control of vectors.

Seven plenary / guest lectures have been arranged comprising of eminent scientists / parasitologists / environmentalist etc. in the field of vector control. This will be followed by practical demonstrations, including standard procedures and staining protocols for chromosome preparations, detection of base-line susceptibility both at larval and adult stages, diagnosis of malaria and other biotechnological tools in the control of vectors.

It is sincerely hoped that outcome of this workshop would inspire the younger generation to take up active research in the area of parasitic diseases and its control.

I am grateful to Dr.N.K.Ganguly, Director - General, Indian Council for Medical Research, New Delhi and President of Indian Society for Parasitology, for suggesting me to organize the workshop and for his constant encouragement. I am thankful to Dr.K.K.Kamboj, Secretary, Indian Society for Parasitology, and Dr.V.P.Sharma, former Director, Malaria Research Centre, Delhi, for their encouragement. I also take this opportunity to thank my colleagues and friends for their co-operation.

Feb, 18th, 1999

Prof. N. J. Shetty
Chairman & Organizing Secretary
13th National Congress for Parasitology

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Part - I

Recent Trends in Vector Control

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RECENT TRENDS IN VECTOR CONTROL

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SUMMARY

This paper describes different methods of vector control including genetic (biotechnological tools), chemical, biological, environmental, integrated vector control and other methods. The possibility of using transgenic species in the future vector control programme is highlighted.

In order to combat vector borne disease, either the parasites or the vector should be controlled. It is generally believed that vector control is more appropriate. The control of vectors faces two important challenges namely - the development of resistance to insecticides and pesticides and the environmental pollution caused due to the constant application of insecticides and pesticides. Therefore, it is desirable to have alternate strategies which do not involve resistance among the vectors. Genetic control or autocidal control is one such method.

The main objectives of vector control is to keep the vector density at the low level to minimise vector-reservoir contact and to curtail the longevity of vector species to interrupt disease transmission. Vector control measures are undertaken where population aggregate for the sake of feeding, resting, breeding etc., particularly during the high density period.

There are several strategies for controlling vectors, including 1) exclusion / quarantine of foreign pests, 2) eradication, 3) plant resistance, 4) biological control 5) chemical control 6) genetic control, and 7) integrated pest management.

A. BIOLOGICAL CONTROL

Biological control agent of insects are generally divided into three groups; parasitoids, predators and pathogens. Parasitoids and predators are most often categorized as naturally occurring biological control agents that are used in pest management systems by conserving established species or by introducing exotic species. In contrast, it is commonly believed that the use of insect pathogens as biological control agents is tried exclusively to their development as microbial insecticides. While it is true that many insect pathogens are currently being used as microbial insecticides and have a great potential for becoming the microbial insecticides of the future.

Of several biological agents, larvivorous fishes are still considered to be the most potential and effective predators for the control of mosquito. The *Poecilia reticulata*, commonly known as guppy can tolerate high degree of pollution and *Gambusia affinis* is a surface feeder and prefers to breed in clean oxygenated water and can be used effectively for the control of both *Anopheles* and *Culex* species.

The toxins of certain spore-forming bacteria viz. *Bacillus thuringiensis* variety israelensis H14 strain and *Bacillus sphaericus* have recently shown great promise as a microbial control agent for mosquitoes. The principal advantages in using microbial insecticides are safety and host specificity. At least five different Orders and eleven different Families of nematodes contain entomopathogenic species (Tanada and Kaya, 1993). Mermithid nematodes exhibit their host specificity. Insects parasitized by mermithid nematodes are often abundant and common in crickets and grasshoppers. Steinernematid nematodes are generally not very host specific and because they kill their hosts within a few days, these species that can be mass produced on artificial media have been commercialized as biological insecticides.

B. CHEMICAL CONTROL

Insecticide research led to the first, "Complete" - victories in combating pest almost 50 years ago with chlorinated hydrocarbons followed by the

organophosphates, methyl carbamates, and pyrethroids - all neuro active chemicals. This golden age of discovery was the source of most of our current insecticides. Muller was awarded the 1948 Nobel prize in Medicine for discovering the insecticidal activity (Muller, 1959).

The very wide spread use of insecticides for pest control is largely a result of their convenience, simplicity, effectiveness, flexibility and economy.

C. ENVIRONMENTAL MANAGEMENT FOR VECTOR CONTROL

WHO expert committee on Vector Biology and Control in 1979 defined environmental management as follows:

"The planning, organisation, carrying out and monitoring of activities for modification and / or manipulation of environmental factors of their interaction with man with a view to preventing or minimising vector population and reducing man-vector pathogen control".

This approach which should be carried out prudently and skillfully is naturalistic and involves an attempt to extend and intensify natural factors which limit vector breeding, survival and contact with man. But these measures have many constraints and limitations, *viz.*

i) Selective applications,
ii) Require high degree of inter-sectoral coordination,
iii) Capital investment of some of the methods is high,
iv) Maintenance is very essential and
v) Active and sustained community involvement.

This is most simple and dependable method of elimination or prevention of mosquito breeding by identifying the active and potential breeding sites of mosquitoes. Environmental management of vectors are much suited for the vectors of urban malaria and dengue / DHF as their vectors mainly breed in overhead tanks, coolers and other man made containers in domestic and peridomestic situations.

i) Drainage

Different species of mosquitoes are known to be associated with varied type of water bodies. It may be impounded rain water, seepage water, natural water sources or man made water courses etc. These can be eliminated by formulating an effective drainage system which will not permit water stagnation and mosquito breeding.

ii) Mosquito breeding associated with the construction of roads / railways

High mosquitogenic potential is generated during the construction of roads and railways and most of these breeding places can be eliminated if proper engineering methods are followed. The breeding places includes burrow pits, culverts, quarry pits etc. Such breeding places need special attention by railway health authorities who may use a combination of various vector control measures in an integrated way.

iii) Irrigation and mosquito breeding

Engineers should incorporate various engineering devices in consultation with public health experts to include an inbuilt system to drain off the seepage water for its better utilization in agriculture. If proper measures are taken, breeding of most mosquito species can be effectively controlled.

D. PERSONAL PROTECTION MEASURES

1. Anti - adult measures

Several personal protection measures are available for providing protection against insect bite. They can be used as supplementary measures in remote and inaccessible areas or against exophilic and endophagic vector species depending upon their feasibility, cost effectiveness and sustainability.

i. Physical method

This include protective clothing's, use of bed nets, screening of windows/ doors etc.

ii. Repellents

These are chemicals that prevent insect damage to plants o animals by rendering them unattractive, unpalatable, or offensive. Thus, repellents include a wide r: ge of chemicals, from volatile substances active in the vapour phase to protect humans against biting flies, and mosquitoes, DMP (Dimethyl phthalate) and DET (Diethyl toluamide) to persistent chemicals such as Bordeaux mixture which act as feeding deterrents to foliage feeders. Many species of ticks throughout the world are serious pests of man and animals. Current methods of protection from attack by ticks depend upon personal protection through the use of repellents.

iii. Space spraying by mist, thermal fogging or ULV spray

Space spraying has been successfully used to control the out breaks of vector-borne diseases such as malaria, dengue, Japanese encephalitis, Western equine encephalitis etc. The space spray is usually undertaken to control the resting population of mosquitoes either by using the natural pyrethrin extract diluted in kerosene oil or malathion during out break situations to interrupt the disease transmission by crisis. This is done in the form of mist, chemical fogging or ULV spray.

2. Anti larval measures

Anti larval measures are used as an adjunct to other methods of control and are rarely used as main method of control except against container breeding species or against those mosquito species which breed in confined or specific small water bodies such as *Aedes aegypti, Anopheles stephensi* and *An. sundaicus*. Antilarval measures can also be tried in an area where vector species are resistant to com-

monly used insecticide or exhibit exophily and exophagy or under those situations where adulticide measures are not cost effective or tend to endanger the environment.

Antilarval measures in tropical countries are mainly used in urban or peri urban areas. These measures can be used in certain specialised situations like mining, irrigation, wells, tanks etc. if they are operationally feasible and cost effective. The basic idea of all antilarval measures is to prevent, reduce or eliminate the breeding places.

i) M.L.O (Mosquito larvicidal oil - Oiling is done in situations where breeding is temporary and permanent measures may not be cost effective. Oiling of a breeding sites kill the larvae by choking their spiracles with oil film and cutting the oxygen supply. It also deter the adult mosquitoes from egg laying.

ii) Paris green (Copper aceto-arsenite) - It has been successfully used in malaria control programme for the control of anopheline and culicine breeding. It is applied as dust or granular formulation.

iii) Abate (Temephos) and Baytex (Fenthion) - Widely used under urban malaria scheme for the control of breeding of anopheline and culicines mosquitoes.

E. GENETIC CONTROL MECHANISMS

Despite the advantage of insecticides for pest control, the use of insecticide has been ecologically unsound, leading to disadvantages such as insect pest resistance, outbreak of secondary pests, adverse effects on nontarget organisms, objectionable pesticide residues and direct hazards to the user and environmental pollution caused due to the constant application of insecticides and pesticides. Therefore, it is desirable to have alternate strategies which do not involve resistance among the vectors. Genetic control or autocidal control is one such method.

The use of insects to control populations of their own kind through the transfer of damaged genetic material represents a new approach that could prove to be useful for the control of a number of major insect pests throughout the world.

The principal requirement for the success of all genetic control methods is the production of sufficient numbers of healthy, competitive (though genetically different) insects and their release in the right place at the right time for them to mate successfully with wild insects. Success will depend on the knowledge of how to rear the species on a large scale, how to sterilize or otherwise genetically manipulate without affecting mating ability and competitiveness and a detailed acquaintance with the general ecology and bionomics of the insect to be controlled (Davidson, 1974).

Translocations

A chromosomal translocation involves the breakage of two non-homologous chromosomes and the reattachment of the broken parts to the wrong partners. During meiosis, in the translocation heterozygotes, the synoptic forces between homologous loci result in the formation of a cross shaped figure (Belling and Blakesles, 1924). It is the subsequent segregation pattern of this configuration which can lead to reduced fertility of translocation heterozygotes and which may be utilized for insect control. Naturally occurring translocations are rare but can be induced either by applying chemicals or radiations.

Translocation heterozygotes have important uses in the development and application of several genetic control mechanisms like sex-sorting systems, translocation homozygotes and compound chromosomes of these translocation heterozygotes especially those which are sex linked can be mass produced and released to introduce sterility into a fertile population thus acting as sterile insect release method agent. There are three important steps involved in the application of translocations in the control of vectors. These include, 1) Synthesis of translocation/s with desired percentage of semisterility, 2) Mating competitive studies between

the translocated males and normal males in the presence of normal females in both laboratory and field conditions, and 3) If the mating ability of the translocated males are higher than the normal males in the above said experiments, then the pilot release experiment would be carried out in selected area/s. A few successful field release studies have been already been undertaken in few species of insect vectors of disease and agricultural pests.

Genetic sexing

In genetic control programme(s), semi-sterile males are released into natural population. Since the females of the insects are potential vectors and cause biting nuisance, they should be eliminated during early development stages by genetic methods. This also help to lower the cost of mass production of males for release purposes.

Genetic sexing mechanisms utilising conditional lethal (eg. Insecticide susceptible gene or temperature sensitive gene) and radiation induced Y-linked translocation and a inversion been carried out for a number of mosquito vectors. These include *An. gambiae* species A; the dieldrin resistant semi-dominant autosomal gene was translocated to the Y-chromosome via radiation induced translocation (Curtis et al., 1976). In *An. albimanus* the genetic sexing systems utilizes propoxur susceptibility as a recessive conditional lethal (a) T (Y;2R) translocation and b) In (2R) inversion. The propoxur resistance allele (dominant) was linked to the Y-chromosome via radiation induced translocation and genetic recombination was suppressed by inversions (Seawright et al, 1978). In *An culicifacies,* dieldrin resistance locus on chromosome III has been translocated to the Y-chromosome and the cytological evidence for the same has been demonstrated (Baker et al., 1981). In *Cx. tarsalis* a genetic sexing strain was synthesised by linking malathion resistance and male determined genes via radiation induced translocations (Mc Donald and Asman, 1982). In *An stephensi,* dieldrin resistance gene is used for the synthesis of genetic sexing strain (Robinson, 1986). In *Cx. quinquefasciatus,* sexing strain was synthesized for the preferential elimination of females during early larval stages. Translocations

were induced which linked the genes for malathion resistance to the male-determining factor T(1M;2)1. Cytological preparations from the above said line revealed a reciprocal translocation between the distal end of the sex-chromosome (chromosome 1) and about the middle of the larger arm of autosome (Chromosome 2) (Shetty, 1987). However, in *Cx. tritaenorhynchus,* a temperature sensitive gene induced by ethyl-methane sulphonate has been combined with sex determining factors as a method of sexing adults (Baker et al., 1978). Genetic sexing strains have also been synthesized in *Ceratitis capitata* Robinson & Von Heemert, 1982)

Sterile insect technique

The Sterile Insect Technique (SIT) is based on the induction of sexual sterility in males through the use of radiation or chemical sterilants and on inundating natural populations with such males (Knipling, 1955, 1959). Such techniques have been extensively used for the control of insect vectors of diseases, insect of veterinary importance and agricultural pest.

The major success has been achieved in the control of the screw worm, *Cochliomyia hominivorax* (Coquerel), a major pest to livestock in the Southern United States. In this case, control is effected through the competition for mating between sterile mass-reared males and the natural population of males. At present the screw worm has been eradicated from the Southeastern United States. The other examples are *Cx pipiens quinquefasciatus, Aedes aegypti, An -albimanus* (Human disease vectors); Mediterranean fruit fly - *Ceratitis capitata* (Agricultural pests); Australian sheep blow fly - *Lucillia cuprina dorsalis* (Live stocks).

Cytoplasmic incompatibility

Incompatibility means that in some way the production of normal offsprings is prevented in a cross between stains of different geographical origin of the same species.

	KA X JB	KB X JA
1	+	+
2	+	-
3	-	+
4	-	-

1) Both reciprocal crosses i.e., KA X JB and KB X JA are fertile (bidirectional compatibility)

2) KA X JB is fertile, KB X JA infertile, **or**

3) KB X JA fertile and KA X JB infertile (unidirectional incompatibility)

4) Both reciprocal crosses are sterile (bidirectional incompatibility)

Sterility in certain crosses between strains of *Cx.pipiens* complex was first reported by Marshall (1938). Laven (1967a) made extensive studies between certain allopatric populations of *Cx.pipiens*. Such an incompatibility which can be either uni or bi directional and which is maternally transmitted has been ascribed to the presence of a rickettsial endo - symbiont, *Walbachia* species in the gonads. Thus, incompatibility is due to the death of the sperm nucleus in an incompatible egg cytoplasm before karyogamy occurs. As a result, no progeny are produced from such incompatible crosses.

This method was field tested with successful results in a small isolated village, Okpo near Rangoon, Burma during a 3 month period and 5-6 generations (Laven, 1967). The released incompatible male strain contained cytoplasm of a strain from Paris, France and the genome of a Fresno, California, strain and resulted in "eradication" of the native Okpo population. This successful field trail helped to generate much interest and enthusiasm in genetic methods of mosquito control. Similar cyto-

plasmic incompatibility exists in *Aedes albopictus* and could presumably be used for genetic control (Kambhapati et al., 1993).

Example : Results of Crosses between strains of *Culex quinquefasciatus* from **Europe** and **India** (Shetty, 1982).

Female Parent	Male Parent						
	Md.	Ma.	Ba.	Mn.	Ca.	Ld.	Mz.
1. Madras					-	-	-
2. Mangalore					+	-	-
3. Bangalore					-	-	-
4. Mandya					+	-	-
5. Cairo	+	+	+	+		-	-
6. London	+	-	-	-	-		+
7. Mainz	-	+	-	-	-	+	

+ Compatibility - Incompatibility

Designation of strain	Origin of strain	Bidirectional compatibility	Unidirectional incompatibility	Bidirectional incompatibility
Md	Madras, South India		Ca, Ld	Mz
Ma	Mangalore, South India	Ca	Mz	Ld
Ba	Bangalore, South India		Ca	Ld, Mz
Mn	Mandya, South India	Ca		Ld, Mz
Ca	Cairo, Egypt	Mg, Mn	Md, Ba	Ld, Mz
Ld	London, United Kingdom	Mz	Md	Mg, Ba, Mn & Ca
Mz	Mainz, W. Germany	Ld	Ma	Md, Ba, Mn & Ca

Refractoriness to disease transmission

The production of genetically defined lines of vector mosquitoes refractory to the development of parasite and thus incapable of transmitting the infection is one possible method of controlling vector borne diseases (Ward, 1963, Shetty et al., 1996).

Apart from selection of malaria refractoriness by old fashion animal breeding technique, ideas have also been presented as to how refractoriness might have been contrived by genetic engineering method (WHO, 1996). These suggestions include the introduction of gene coding for a transmission blocking single chain antibody (Winger et al., 1987) namely an antibody against *Plasmodium* gamete, zygotes or oocyst which are produced in or on the stomach of the mosquito.

Molecular genetics and transgenic insects

Recent techniques in biotechnology provide tremendous potential for improvement of the effectiveness of natural enemies against insect pests. This could range from the development of genetic sexing strains to engineering a disease and pesticide resistance for beneficial insects.

The term 'Transgenic' indicates the incorporation of a 'desirable gene' into the germ line of the organism, which is stably propagated / transmitted to the offspring. The successful use of genetic engineering for the improvement of insect parasitoids is a multistage project which involves the integration of a number of distinct techniques. Initially, traits that are desirable need to be identified and characterized. Next, genes involved in expression of the desired trait must be cloned and characterized. Finally, these genes must be suitably modified and successfully introduced into the target organism's DNA such that the desired trait will be transmitted in a Mendelian fashion.

Attempts to transform non-Drosophilid insects have been considerably less successful. Thus far, transformation of 3 species of mosquitoes have been reported - all using the 'p-element' vector pUChs neo (which carries the bacterial gene for neomycin resistance as selectable marker). Current interest in control of insect - borne diseases by genetic manipulation has resulted in the use of molecular techniques that may extend to vector species. Recent progress in the study of vector competence and insecticide resistance have begun to stimulate interest in detailed molecular analysis of physiological processes that might be manipulated in transgenic strains. Unfortunately, low frequency of detection of plasmid insertions and relative instability from generation to generation have prevented the routine production of transgenic mosquitoes.

However, introduction of DNA constructs into mosquitoes cell is readily accomplished, with both transient and stable transformation of *Ae- albopictus* and

Ae- aegypti made possible (Monroe et al, 1992, Shotkoski & Fallen, 1993).

Some of the proposed / reported delivery systems (for genetic manipulation of vector species) include :

- Microinjection of DNA into eggs
- Microprojectile bombardment
- Cationic liposomes as delivery systems
- Transduction using a recombinant virus delivery system (e.g., Sindbis virus developed by Mulligan, 1993)
- Introduction of genetically altered cells into embryo

Screening and detection of transformants :

Transgenic mosquitoes may be detected by :

- Including a gene for a visible marker in the recombinant DNA construct (e.g. eye colour)
- Use of dominant selectable marker (such as DHFR gene which has been amplified approximately 1200 - fold during selection for methotrexate resistance, Shotkoski & Fallon, 1991)
- Genes which confer resistance to insecticides
- Use of **PCR** to screen for presence of genetic construct

Assuming that efficient routine transformation of mosquitoes can be achieved - there arises the question of what genes to be incorporated into them. Some of the strategies devised are based upon work in other systems/genetic models. Molecular analyses of hematophagy and vector competence, and other genetic and physiological processes can be exploited as targets in transformational studies. From genetic studies it has been demonstrated that even a single gene can profoundly affect vector competence - identification of such loci in certain strains, which confer refractoriness to infection by pathogens can be cloned, characterized and subsequently exploited to control transmission of disease. Mutation in these genes (which probably encode products essential for parasitic growth and development) may confer parasite - refractory phenotypes.

Although characterization of the natural bases for vector competence might

provide important insight for vector control, present efforts with transgenic organisms would be facilitated by the availability of a refractory gene with a dominant phenotype, that is completely penetrant and that shows no variable expression. In addition, because one of the goals of manipulating genes involved in vector competence is to increase the prevalence of the gene in wild populations, a parasite resistant gene should have no deleterious effects on the organism.

However, lack of a naturally existing gene which exhibits all desirable properties have led several investigators to propose the synthesis of genes with antiparasitic activity. Possible antiparasitic coding regions included antisense RNA's to target viral pathogens and cloned genes that express antiparasitic antibodies.

Another important aspect that needs to be dealt with is that of stable maintenance of a genetic construct within the organism, enough to be propagated from generation to generation.

There are two possible ways for stable maintenance of a recombinant - DNA construct within the organism.

i) The genetic construct may become integrated into the genome via a recombination event
ii) Construct may be maintained as a separate self replicating entity that faithfully replicates and segregates from generation to generation

In the former case, however, the number of illegitimate integration exceeds homologous recombination, and may result in considerable variation in the expression of the gene construct in the transferred cells.

Integration via transposable elements also occurs by illegitimate recombination, the event being mediated by 'transposase' that acts on the ends of the transposable element. This generally results in only a single copy integrated per site without re-

arrangement of the genes carried on the transposon.

However, site specific recombination (which is preferred) and which mediates the recombination between DNA molecule that contain specific target sequences, requires a specific enzyme - a recombinase.

Expression of genes on recombinant DNA constructs, introduced into mosquito cells depends upon the availability of suitable transcriptional promoters, terminators, RNA processing signals, translational control sequences and so on.

Since only few of these have been identified and their functions characterized in mosquito, *Drosophila* promoters have been introduced into mosquito tissue culture cells and in some cases, expression detected during introduction of either heat-shock or metal ions.

Moreover, as many of the signals that control various aspects of gene expression, such as RNA processing and initiation and termination of translation, are conserved through out eukaryotes, they could be expected to function in mosquito regardless of their origin.

Drawing upon the latest studies and techniques provided by molecular biology, vector biologists hope eventually, to develop transgenic strategies for control of disease transmission. Some of the difficulties faced during such a programme are,

Requirement of more information and experimental studies on:-

- Appropriate time of DNA introduction (preferably early cleavage stage, nuclei are actively replicating and prior to cellular membrane formation)
- Appropriate region / site of DNA introduction (preferably cells / region which ultimately becomes the germ line of organism)
- Site of insertion of 'p-element' (due to illegitimate recombination, insertion can have significant effect on expression of gene both in terms of level and tissue specificity of expression, proximity of enhancers, and regulatory

sequences)

- Identification of heterologous / endogenous mosquito transposable elements that may function in the vector species; detailed knowledge and information regarding molecular genetics of appropriate transposon used
- Behavioral patterns (e.g. egg laying behavior, kinetics of chorion hardening, pole cell formation considered, rate of survival of embryo after microinjection etc.

Since considerable research in the area of molecular biology and bio-technology is being carried out among the insect vectors, it is hoped that such desirable gene(s) will be incorporated into the vector population for field release studies in the future genetic control programmes.

Other mechanisms

The phenomenon of "meiotic drive" has been recognized for its potential for the deliberate introduction of genes into population. Sandler and Novitski (1957), first used this term to describe those occasions where unequal recoveries of alleles from heterozygotes occurred. An individual of constitution Aa producing more "A" gametes than "a" or vice versa is said to exhibit this phenomenon, and the explanation is that the one chromosome of a pair is "Driven" more than the other at meiosis and is consequently more likely to end up in a functional gamete. *Segregation - Distorted (SD)* on chromosome 2 of *Drosophila* and MD locus in the mosquito is an example of Y-linked meiotic drive. Males heterozygous at this locus may pass the *SD* bearing chromosome to more than 95% of their offspring.

F. INTEGRATED VECTOR CONTROL

The integrated vector control may be defined as the application of one or more than one vector control method. Simultaneously or consequently in a given area to control vector borne diseases when available options are selected on the basis of epidemiological paradigm, vector behaviour, human behaviour and environmental aspects, it become selective vector control. This approach is quite appropriate but requires effective planning, technical, competence, managerial skills

and sound understanding of vector and its environment.

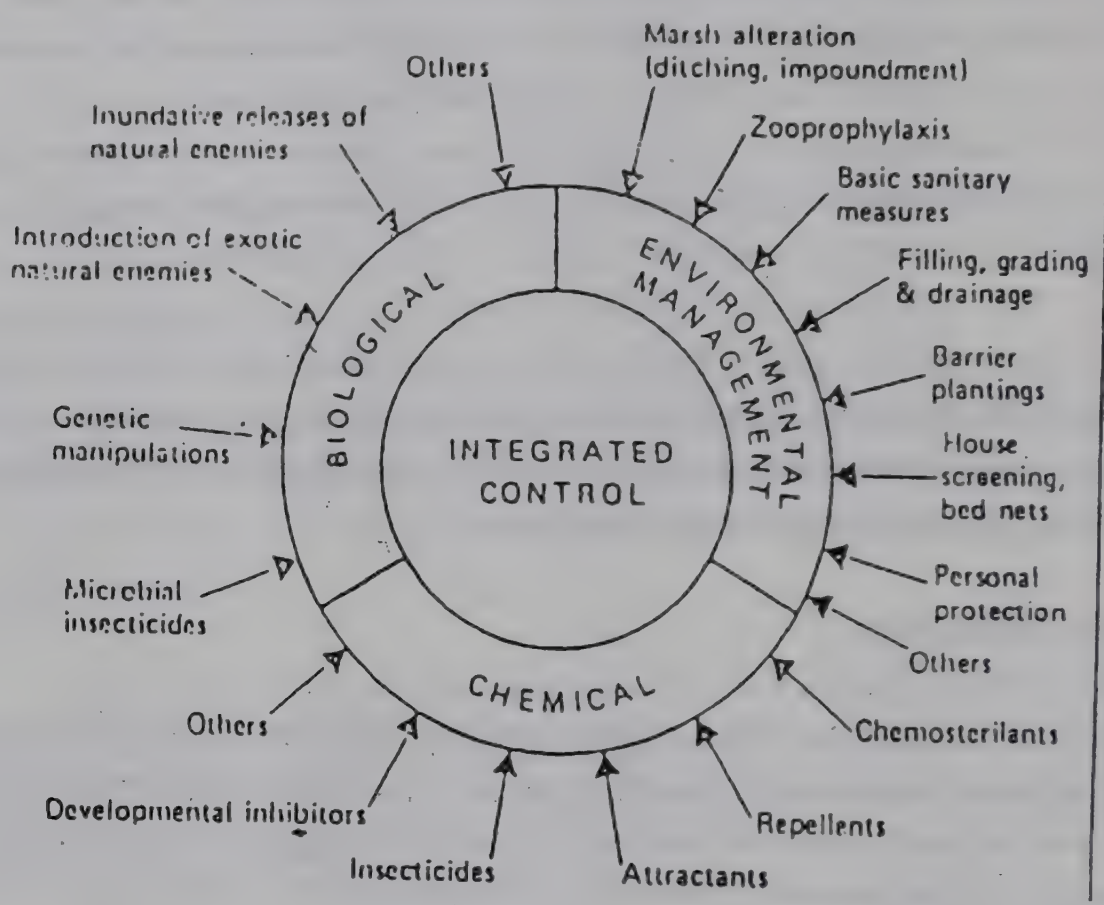

Fig. 1

Public health education and community participation in the control of vector borne diseases

The role of public health education is vital for the effective implementation of vector control measures, in respect of vector-borne diseases, as the problem mainly revolves around man and his environment. The aim of the health education for the control of vector-borne diseases like malaria, filariasis, Japanese encephalitis, Dengue/ DHF and kala-azar should be to familiarise and motivate people by highlighting the

following aspects of the disease :

- Causation and mode of disease transmission.
- Awareness about the early signs and symptoms of the disease.
- Educating the masses to co-operate with the public health workers for the early diagnosis of disease by clinical symptoms and laboratory diagnostic tests.
- Usefulness of taking proper and adequate treatment of disease.
- Knowledge about the breeding, feeding and resting behaviour of vector species.
- Usefulness of insecticidal spray for the control of mosquito / sandfly borne diseases to bring down vector density and to curtail their longevity to interrupt disease transmission.
- To restrain local inhabitants from mud plastering of walls of houses, cattlesheds etc. for a minimum period of two months after the spray to retain the residual effect of insecticide for the effective control of mosquitoes and sandflies.
- Personal prophylactic measures by using ordinary impregnated bednets or repellents to prevent mosquito / sandfly bite.
- To educate masses to sleep on the cots/ benches instead of on floor to prevent sandfly or flea bite.
- To undertake bio-environment measures to reduce the breeding, resting and feeding places in and around human habitations, cattlesheds etc.
- Use of mass media like radio, television, cinema slides, newspapers, posters and film shows, etc. Involvement of social workers, teachers, school children, public health workers voluntary health organisations / resident welfare associations should be involved for disseminating the above given information by holding group meetings or by inter-personal, communication, health exhibitions etc. to get rid off/reduce mosquito breeding.

Community participation

WHO Alma Ata Declaration (1973) envisaged that community participation should be considered as a crucial component of Primary Health Care (PHC). The

idea of community participation was developed with the fond hope that it would bring communicable diseases under control to reduce mortality due to communicable diseases and to improve quality of preventive care, it was considered advantageous to encourage participation of people. Involvement of community for the success of any vector control programme assumed still greater significance as the problem revolves mainly around man and his environment. The community will perceive the impact of control measures which will stimulate their active involvement in PHC socially, culturally and technically.

Consequent to the resurgence of kala-azar in Bihar in 1977, plague in Maharashtra and Gujarat in 1994 and Dengue / DHF in Delhi, Haryana and Punjab during 1996, it was observed that the public health education about vector-borne diseases is poor and community participation was practically nil. It is felt that for the control of vector-borne diseases, community may be motivated to co-operate and participate for the effective implementation of vector control strategy. After motivation, the community would be able to extend their full co-operation in getting their dwelling units, cattlesheds etc., sprayed with insecticide and should restrain from mud plastering the insecticide treated surface for a minimum period of two months for the retention of residual effect of insecticide. Besides, the community may be motivated to undertake bio-environmental measures like removal of garbage from in and around the houses, pigsties, cattlesheds, filling of all cracks and crevices. The shelters should be made more ventilated and lighted to prevent the breeding, resting and feeding of vector species.

For the success of vector control programme, there has to be frequent interactions between the health workers and the people so that they may accept the control programme as the "People's Programme" and only this approach will be fruitful for the effective implementation of vector control strategy vis a vis control of vector-borne diseases in various parts of the country.

CONCLUSION

Armed with the ever-improving tools available for biochemical and molecular genetic analysis, impressive progress is being made in the field of insect vectors. It is only through the combined efforts of individuals with varied talents and training that we will be able to address the problem and device effective solutions.

Part - II

Vector Surveillance and Diagnosis of Human Malaria and Filariasis

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A. VECTOR SURVEILLANCE

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During the recent years, many diseases specially vector borne have re-emerged in many parts of the world. In India, malaria, filariasis, Japanese encephalitis, dengue etc. are prevalent and deaths due to these are the cause of concern. Vector control imparts the major part to contain these diseases and accordingly vector surveillance is very much necessary. Some of the important aspects of vector surveillance are given below.

Methods for estimating adult and larval mosquito density

Adult Mosquito : Adult mosquitoes can be collected from different surfaces by a test tube or sucking tube. Sucking or aspirator tube collection is most widely used for mosquito collection. The tube is made up of glass or plastic having a length of 30-45 cm with 8-12 mm diameter. A piece of mosquito netting is fixed on a small piece of lesser diameter rubber tubing. This is placed at one end of the larger tube. A long rubber tube (about 50 cm) is pushed over the end provided with the netting. The other end of the tube may be kept slightly curved. The resting or feeding mosquitoes when detected in torch light are sucked one after another and then transferred in to test tubes.

1. Man Hour Density (MHD): This is expressed by the following formula

$$\frac{\text{No. of mosquitoes collected X 1 hour}}{\text{No. of collectors X No. of hours spent}}$$

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2. Per Room/Shed Density : This is also called total catch and generally done by space spray in a unit room preferably closed. Spraying is done with 0.1-0.2% pyrethrum extract @ 30-60 ml/1000 cubic feet.

3. Man-mosquito contact (Man biting rate) : Man-mosquito contact can be assessed by biting or landing rate (MBR). This is determined by counting the number of mosquitoes collected while biting or landing on human or animal bait per night or unit time.

4. Parity Rate : This is obtained by simple detection of parous females on the basis of tracheolar coiling (Detinova technique) or number of dilatations on the pedicles of the ovarioles (Polovodova technique). Parity rate can be expressed as

$$\frac{\text{No. of parous females encountered}}{\text{No. of female mosquito dissected}} \times 100$$

5. Gonotrophic condition : Collected female mosquitoes can be classified as unfed (UF), fed (F), half gravid (HG) and full gravid (FG). This parameter is necessary to assess the efficacy of anti-adult operation undertaken.

6. Human Blood Index (HBI) : This is also known as anthropophilic index. This is expressed as percentage of mosquito blood meal found positive for human blood. This index helps in determining the extent of man vector contact.

7. Collection of mosquitoes using traps : Various types of traps like Magoon, window, Light trap (CDC) are used for studying mosquito behaviour. This is expressed as number of mosquitoes collected (species wise) per night per trap.

Larval Collection Methods

Mosquito larvae are collected from various breeding habitats and can be collected at any time during the day. Collections are done by the following methods :

(a) Dipping : This method of larval collection is mostly used in the field. The collecting equipment like enamel bowl, ladle (about 9-10 mm in diameter) are dipped at the edges of the breeding sites at an angle of 45°. Larval density is calculated by the average number of larvae collected per dip.

(b) Netting : Larvae may be collected from large stretches of water bodies like streams, ponds, wells. The net consists of a ring of iron frame (20-25 cm in diameter) to which a nylon or muslin cloth net is attached measuring about 10 cm long. A wooden long handle is attached with the ring. The net is held at an angle of 30°. Larval density is calculated per net.

(c) Pipetting : Larvae can also be collected using small pipettes from shallow breeding sites like tree holes, hoof prints etc.

For *Aedes* Survey : Larval indices are main parameters for *Aedes* survey

i) **Container index** : Number of containers found positive per 100 containers checked

ii) **House index** : Number of houses found positive per 100 houses checked

iii) **Breteau index** : Number of containers found positive per 100 houses checked

MONITORING OF SUSCEPTIBILITY STATUS OF MOSQUITOES :

Any vector borne disease can be controlled only when the respective vectors are effectively controlled. The use of insecticides are the mainstay of vector control throughout the world. Practically, resurgence of many diseases specially malaria is seen recently due to the development of resistance to DDT against vector mosquitoes. In this scenario, monitoring of the susceptibility status of insecticides to vector mosquitoes is an integral part of control programme. World Health Organization (WHO) provides specific guidelines from time to time on this aspect based on the results obtained from various studies carried out in different parts of the world. Details of adult and larval susceptibility test are given below. Susceptibility kits are available from WHO. Detailed technique has been described in *WHO Technical Series No.*443, 1970.

Adults : Adult mosquitoes are tested by WHO test kit in which the following materials are required :

Materials

a) 20 plastic holding tubes - each having 125 mm length and 44 mm diameter

- 8 tubes with red dot - exposure tubes
- 2 tubes with green dot - control tubes
- 10 tubes with green dot - holding tubes

b) 20 slide units with a screw cap on either side provided with a 20 mm filling hole.

c) Plain paper sheets for lining of holding tubes.

d) 20 spring wire clips; 8 copper clips for exposure tubes and 12 silver clips for holding and control tubes.

e) Glass sucking tube (aspirator).

f) Log probit paper.

g) Adhesive tape

h) Insecticide impregnated papers like organochlorine, organophosphate, carbamate and synthetic pyrethroids along with control papers.

Methods

Healthy freshly fed female mosquitoes collected from the study area are exposed for a period of one hour under laboratory conditions. Mortality of the mosquitoes are recorded after 24 hours. The following methods are followed :

a) In each holding tube a piece of white paper is placed inside and a silver spring clip is put for holding the paper.

b) About 15-25 mosquitoes are kept in each holding tube with the help of sucking tube and the tubes are kept in upright position for 1 hour. The moribund mosquitoes are discarded.

c) In the exposure tubes different insecticide impregnated papers are kept. In the control tubes control papers with solvent oil are also kept. Copper clips are put to keep the papers in position.

d) With the help of sliding unit the mosquitoes are transferred in the exposure and control tubes respectively from the holding tubes.

e) The tubes are kept upright up to one hour.

f) The mosquitoes are transferred to the holding tubes after the exposure. Glucose soaked cotton pads are kept at the top of the wire mesh and kept for 24 hours at controlled laboratory conditions.

g) Mortality rate are calculated after the exposure. At least 2-4 replicates should be made for each type of insecticide.

h) The tests are considered valid when the control mortality is within 5-20% which can be corrected by Abott's formula; otherwise the tests should be repeated.

$$\text{Abott's formula} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 \% \text{ control mortality}} \times 100$$

Interpretation : Percent mortality from each set is obtained which can be put in log-probit graph paper. Regression lines can be drawn putting the LC_{50} and LC_{95} values.

Precautions : The impregnated papers should be properly resealed after each use. The impregnated papers can be used about 20 times and up to 3 weeks after removal from the packet. The impregnated papers should be kept in dark cool place and should be used within the shelf life of the papers.

Larvae : Susceptibility status of immature stages of mosquitoes can be determined by exposing late 3rd or early 4th instar larvae to various insecticide dosages in 500 ml glass container. The reading is taken after 24 hours exposure period. Mortality is recorded by the method recommended by WHO.

Following criteria are considered for establishing the susceptibility status.

Range of mortality	Status
• Between 98-100%	Susceptible
• Between 80-98%	Tolerant (requires verification)
• Below 80%	Resistant

B. DIAGNOSIS OF HUMAN MALARIA AND FILARIASIS

HUMAN MALARIA

Malaria is one of the major public health problems in India. Recent data shows that though the malaria cases in the country are at the level of 2 to 3 million per annum but *Plasmodium falciparum* cases have raised upto 40% of the total cases. Moreover, there is a progressive increase of chloroquine resistance against this parasite. This needs proper treatment of malaria and thus it is imperative to determine the species of parasites in a patient. Since the slide examination is still the main method for diagnosis of malaria, it is important that the slides are properly made, stained and examined. In such conditions, it is fairly easy to detect the malaria parasite and the species. Early detection and prompt diagnois can interrupt transmission and may save many lives.

Method of staining

The microscopic slides used for making the smears should be of good quality (75 mm X 25 mm X 1.5 mm). The slides are cleaned by boiling with soap solution or any other detergent. Subsequently, they are scrubbed and washed thoroughly in running water so as to remove all traces of detergent and grease.

The tip of the middle or ring finger of the left hand is cleaned with spirit swab and allowed to dry completely before pricking. Two drops of blood are collected, one for thick smear and the other for thin. The thick smear is made in such a way that edge of the smear is approximately 1 cm away from the edge of the slide. The

distance between the edge of the thick and thin should also preferably be 0.75 cm. The thin smear is fixed with Methanol. Care should be taken so that no methanol is allowed to come in contact with the thick smear. The smears are stained after complete drying.

Giemsa stain : The stock stain is prepared from Giemsa powder (preferably E. Merck, Germany)

Giemsa powder	-	3.8 g
Glycerol (pure)	-	250 ml
Methyl alcohol (pure)	-	250 ml

The stain is prepared by mixing all the ingredients in a porcelain mortar. The prepared stock solution can be filtered and should be kept in a bottle.

Dilution of stain : Stock solution must be diluted at appropriate amount. Generally, 5% fresh solution is prepared for staining the smears. A buffer solution of pH 7.2 can be used for dilution. The buffer is prepared as follows.

Potassium dihydrogen phosphate (KH_2PO_4)	- 0.7 g
Di-sodium hydrogen phosphate (Na_2HPO_4)	- 1.0 g
Distilled water	- 1 litre

JSB Stain (Jaswant Singh and Bhattercharjee, 1944)

JSB stain consists of solution-I and solution-II.

Solution-I : Make by thoroughly dissolving the following ingredients

Methylene blue (preferably E. Merck, Germany)		- 0.5 g
Sulphuric acid (H_2SO_4)	1%	- 3 ml
Potassium di-chromate ($K_2Cr_2O_7$)		- 0.5 g

Di-sodium hydrogen phosphate (Na_2HPO_4)	- 3.5 g
Distilled water	- 500 ml

The resulting mixture is heated to slow boiling in a reflexing condenser for one hour. The stain is allowed to cool at room temperature and should be kept in a closed bottle.

Solution-II : Prepared by dissolving 1 gm of yellowish aqua Eosin powder (preferably E. Merck, Germany) in 500 ml of distilled water. Both the solutions should be kept for couple of months to mature before use.

Buffer solution is prepared by dissolving the following ingredients and adjust the pH at 6.8.

Potassium hydrogen phosphate ($K_2H_2PO_4$)	- 0.752 g
Di-sodium hydrogen phosphate (Na_2HPO_4)	- 3.5 g
Distilled water	- 2 litres

The fixed slides are dipped into solution-II for 2-3 seconds (1 to 3 dips) and subsequently washed thoroughly in buffer water. The slides are then dipped in solution-I for about 45 seconds (20 to 75 seconds). It is again washed gently and thoroughly in buffer water. After drying, the slides are examined under an oil immersion lens (X 100). A slide should be declared negative after examining either 200 thin or 100 thick smears.

Thin Smear Examination

In properly made thin smear the parasites are seen with detailed morphology. In disputed cases, species of the parasites should be diagnosed based on thin smear examination. In this method loss of parasites is not possible.

Plasmodium vivax

All the different stages of erythrocytic cycle in *P. vivax* are likely to be seen in the smear. The presence of schizonts is dependent on the time of schizogony. In ring stages, it is sometimes difficult to differentiate from *Plasmodium falciparum*. The rings are usually thick specially the cytoplasm. At this stage, there is no appreciable enlargement of the erythrocytes. In the trophozoite stage, the parasite is fairly amoeboid. There is appreciable increase in size of the infected erythrocytes. The presence of Schuffner's dots in the erythrocyte cytoplasm is very characteristic of *P. vivax* infection. Schuffner's dots are also present in schizont and gametocyte stages. In schizonts host cells are enlarged almost twice the size of the normal cells. The pigment is usually concentrated in the middle. Merozoites varies from 12 to 24 in number. At the gametocyte stage, the vacuole of the parasite disappears and pigments are found scattered in the cytoplasm. The host cells also enlarge.

Plasmodium falciparum

In the peripheral smears, only rings and gametocytes are seen. The other stages of trophozoites and schizonts are usually absent in the peripheral smears. In the early rings, the cytoplasm is usually very thin and hair-like. They are present in different forms, viz., marginal, tenue and those with double and multiple chromatins placed side or lying in opposite poles.

Multiple infection is also seen in *P. falciparum* infection. The presence of Maurer's dots is characteristic of *P. falciparum*. These dots present on the erythrocyte are coarse and usually few in number. The gametocytes of *P. falciparum* have got characteristic shape of a sausage. The stretched erythrocyte outlines can be seen in most of the parasites. The pigments have a tendency to accumulate at this stage.

Plasmodium malariae

This parasite is very seldom found in patients because of its low prevalence. They can be identified very easily by their characteristic features. The rings are quite

stocky and solid. The cytoplasm is thicker than in vivax. The pigments can be seen in cytoplasm even in ring stage. Majority of trophozoites are band shaped. Coarse blackish pigments can be seen in the cytoplasm. There is no enlargement in the erythrocyte containing the schizont. In fully mature stage, merozoites are 8-12 in numbers. The pigments are concentrated in the centre giving an appearance of rosette.

The gametocytes are round in shape like in vivax. But the host cells are not enlarged at all. They appear to be smaller in some of the parasites. Blackish pigment granules are found scattered in the cytoplasm. They can easily be identified because of the round shape and size.

Thick Smear Examination

Thick smear examination for malaria parasite was introduced by National Malaria Eradication Programme. In fact, thick smear is made of many layers of thin smears. With certain amount of special expertise it is possible to detect the parasites quickly. Sometimes it is difficult to determine the species of parasite. Parasite morphology is distorted in thick smear and loss of parasites is possible.

Like thin smear, the parasites are easily identifiable in thick smear. It is essential to associate the pink coloured chromatin with blue cytoplasm of each parasite for identifying the ring stage. Detection of the presence of mixed infection of two species may also be difficult in thick smear. The advanced stages of *P. vivax* as well as *P. falciparum* gametocytes could be identified easily in thick smear.

Parasitaemia in blood smears

Thick smear : Infected erythrocytes (RBCs) are counted in relation a predetermined number of leukocytes (WBCs) and average of 8,000/µl is taken as standard.

300 WBCs are counted in 100 X field. If more than 10 parasites are present, the following formula should be applied for determination of parasitaemia.

$$\frac{\text{No. of parasites}}{\text{No. of WBCs counted}} \times 8{,}000 = \text{No. of parasites/ µl blood.}$$

If parasites are less than 10, about 500 WBCs should be counted.

Thin smear : Determination of percent parasitaemia is essential for some specific purpose. The number of infected red cells (and not number of parasites) in 10,000 RBCs is calculated to percentage.

The '+ system' is less precise as variation in the thickness of the thick film result in false variation in parasites count.

\+ = 1-10 parasites per 100 thick fields
++ = 11-100 parasites per 100 thick fields
+++ = 1-10 parasites per thick field
++++ = >10 parasites per thick field

Recent advances in diagnostic methods in malaria

QBC System : Becton-Dickinson's Quantitative Buffy Coat (QBC) method involves spinning of blood in special capillary tubes precoated with Acridine Orange (AO) in which parasitetic DNA is stained with AO and a small precision moulded plastic float presses the parasitised red cells against the wall of the tube where they can be viewed under UV light microscope.

Dip-stick method : 'Parasight-F' is a new dip-stick method for the detection of falciparum malaria. In this method, a dual antibody incorporated in a paper strip captures the histidine-rich protein-II antigen (HRP-2) of *P. falciparum*. Diagnosis is made for a pink coloured line on the strip.

Immunochromatographic test (ICT) : The kit consists of a cardboard device with opposable faces. One face has a membrane test strip with a line of immobilised antibody conjugated to visible colloidal gold particles. The target antigen for this test is *P. falciparum* histidine rich protein-2 (PfHRP-2), a water soluble protein synthesized by the parasite and released from the infected erythrocytes. The system has an affinity purified sheep polyclonal antibody specific against the immunodominant repeating sequence from PfHRP-2 molecules. 10 µl of patient blood was collected in EDTA coated capillary tubes and was added to the fibrous pad at the base of the test strip. Then running buffer was added in three specific sites. The blood and gold labelled antibody mixture thus released from the pad. When blood was cleared from the strip with buffer, the card was folded and the result is read through the viewing window. In positive samples, pink coloured bands were formed at the site of the strip within 4-5 minutes. In the negative samples no such bands were seen. A control band was also seen in each case to indicate the validity of the test.

Nucleic Acid Probe

DNA Probe : The eukaryotic genome contains highly repetitive species-specific sequences. The ability to clone such DNA regions provides a means to produce a new array of diagnostic reagents based on DNA-DNA hybridization procedure. The binding of a DNA probe to its specific target DNA is usually detected by tagging the probe with a radiolabel (P^{32}) and exposing the hybridized DNA to an X-ray film.

Since most genomic DNA probes contain repeats of the 21-base pair sequences, synthetic oligo-deoxynucleotides which can be radiolabelled with P^{32} have also been employed as a diagnostic probe.

RNA Probe : DNA probes can be constructed which are specific but are not very sensitive in detecting low grade parasitaemia in patients. A rRNA probe should be in a better proposition, in that there are between 0.2 to 1.o pg. of RNA per parasite compared to 0.02 pg. of DNA. A simplified procedure which by directly applying the blood sample onto nitro-cellulose membrane and detection of parasite is done

using a single 21-mer oligonucleotide probe complementary to a stem-loop region of the parasite small sub unit RNA. 50 parasites could be detected in 50 µl of blood after 1 hour of autoradiography.

PCR : One of the most recent exciting developments in the field of recombinant DNA technology has been the Polymerase Chain Reaction (PCR). By this method, it is possible to synthesize *in vitro,* millions of copies of specific target DNA sequences. In malaria, PCR opens the prospect of increasing sensitivity for detection as well as identifying genes involved in drug resistance. Using the nonisotopically labelled probe following PCR amplification, it is possible to detect <10 parasites/10 µl blood (specific for *P. falciparum*).

LDH Based Assay : Recently, certain key enzymes of the parasites have been explored as a source of diagnostic antigen. One such enzyme is the Lactate Dehydrogenase (LDH) of malaria parasites. The test comprises a nitro-cellulose paper strip coated with anti-plasmodial LDH antibodies which is incubated with drops of haemolysed blood followed by the histochemical staining of the parasite LDH. The appearance of a violet dot indicated a positive reaction.

Serological Tests : Serological methods of diagnosis of malaria have come of practical value since 1962. Generally, it is used for : i) detection and measurement of specific malaria antibodies and ii) detection of scanty parasitaemia. Several methods are applied which is given below :

Test	Current application	Source of Ag	Ab identified
Immunofluorescence	Epidemiological studies, research and aid to diagnosis	Erythrocytic schizonts	IgG, IgM and IgA
Indirect Haemagglutination (IHA)	Epidemiological surveys	Ag coated erythrocytes	IgG -
ELISA	Epidemiological and aid to diagnosis	Soluble Ag	IgG, IgM
Radio-immuno assay (RIA)	Research	Soluble Ag or Ab	IgG, IgM

Detection of Microfilariae

In Blood : Thick blood examination is the best method for diagnosis of microfilariae. It is important that the time of blood collection should be closer to the peak activity time of the microfilarial species and strain. About 20 µl blood is required to make a thick film. The slide should be clean, evenly spread, dried and carefully dehaemoglobinized and stained. Staining is done preferably with JSB.

Provocative Test : This method is applied when a patient is suspected of filarial infection. In this test, 100 mg of diethylcarbamazine citrate [DEC] is administered orally (adult dose; 50 mg for children). The peripheral blood is collected after 30-45 minutes of DEC administration. DEC provokes the microfilariae in the lung capillaries to invade the peripheral blood.

Concentration Technique : This is the most sensitive method of microfilarial detection. Anticoagulated venous blood samples of 1-10 ml is filtered through Millipore or Nuclepore membrane filters of 3-5 µm porosity. The blood is haemolysed with Teepol. The filter is washed three times with normal saline and then fixed with formal saline (1 part of formalin + 9 parts normal saline). Wash twice with distilled water. Blow air through the filter. The dried filter paper is immersed in hot haematoxylin for 5 minutes. 'Blow' briefly in gently running water. Dry on a clean microscope slide. Cover with immersion oil and a coverslip. Examine under low power lens. This is an expensive method and should be done for special cases only.

In hydrocele fluid or urine : Sometimes microfilariae are absent in blood but present in hydrocele fluid or urine. The samples are processed and examined using the concentration technique.

Identification of Human Lymphatic Filarial Worms : In India *Wuchereria bancrofti* and *Brugia malayi* are present. The microfilariae of these species can be identified based on the following characters.

	Wuchereria bancrofti	*Brugia malayi*
Mean length (μm)	290	222
Cephalic space (length : breadth)	1:1	2:1
Sheath colour	Unstained	Pink
Nuclei	Regularly spaced and separately situated	Irregularly spaced and overlapping
Tail	Single row of nuclei do not reach the tail end	Single row of nuclei reach the tail end
Terminal nuclei	None	2 nuclei closely spaced which clearly bulge the cuticle
Innenk" rper length (μm)	34	30.7
Appearance in blood film	Smoothly curved	Kinky

Part - III

Vector Cytogenetics and Related Techniques

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VECTOR CYTOGENETICS AND RELATED TECHNIQUES

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Introduction

Mosquitoes belonging to the order of two-winged flies knows as Diptera, which include houseflies, stable flies, blue-bottles, tse-tse flies and many others. Mosquitoes themselves belong to a sub-order of Diptera known as the Nematocera.

The Culicidae are divided into three sub-families; the Anophelinae including the well known genus *Anopheles*, many species of which are responsible for the transmission of malaria; the Toxorhynchitinae, the enormous larvae of which eat other mosquito larvae; the culicinae which, with about 2000 species are further divided into two tribes : the Culicini and the Sabathini. The Culicine mosquitoes include well-known genera as *Culex*, *Aedes*, and *Mansonia*.

The more than 2,500 species of mosquitoes have been put in 33 genera.

Mosquitoes are excellent research material for conducting cytogenetic and genetic experiments in laboratories because of short life cycle, high reproductive potential, low chromosome number, high genetic variability and comparatively easier maintenance.

An attempt is being made to describe in the present study, with respect to the preparation of polytene chromosomes from salivary gland nuclei salivary gland nuclei and ovarian nurse cells ; mitotic and meiotic chromosomes, banding techniques, etc.,

DIFFERENCES AMONG VARIOUS DEVELOPMENTAL STAGES OF *ANOPHELES, AEDES AND CULEX.*

	ANOPHELES	***AEDES***	***CULEX***
EGGS	Eggs are laid singly on the surface of water. No. of eggs laid at a time varies between 40-100. Eggs are boat shaped.	Eggs laid singly in moist soil and hatch when flooded. Eggs are spindle-like. No. of eggs laid at a time varies between 200-400.	Eggs laid on surface of water in a mass called the egg raft. Eggs are cigar shaped. No. of eggs laid at a time varies between 200-400.
LARVAE	Floats horizontally on surface of water. Has a raised chitinous plate bearing 2 spiracles for breathing air on 8th abdominal segment.	Floats in water obliquely with head lower most. Has a respiratory siphon that is relatively short and stout and bears only one pair of hair tufts.	Floats on water obliquely with head lowermost. Bears tubular chitinous long respiratory siphon on 8th abdominal segment for breathing.
PUPAE	Bears relatively short and broad respiratory siphons on the cephalothorax. It is greenish. Each paddle bears one long and one short bristle.	Bears long respiratory siphons on the cephalothorax.	Bears long respiratory siphons on the cephalothorax.
ADULT	Has a pair of compound eyes and a pair of antennae. Antennae are plumose in males and sparsely feathered in females. The palpi situated on both sides of proboscis are about as long as the proboscis in the male and female.	Parts are similar as *Anopheles* adult.	Parts are similar as *Anopheles* adult except palpi which is shorter in females.
RESTING POSITION	Rests with body making an acute angle with surface. Body is not hump backed when at rest.	Rests with body parallel to surface. Body is hump backed when at rest.	Adults rests with body parallel to surface. Body is hump backed when at rest.

A. MITOTIC CHROMOSOMES

1. For mitotic chromosomes the brain of 4th instar larvae is an adequate source. (Good preparations are often made from late 4th Instars, approximately 6-12 hours prior to pupation). Larvae are pretreated with Colchicine (0.1%) for 2 to 5 hours.

2. Larvae for dissection are removed from their rearing pans using an eye dropper and placed on blotter to remove excess moisture and then transferred to a drop of dissecting medium on a slide. (Dissection is carried out under stereo dissection microscope at 30X magnification using 5% diluted carnoy solution as a medium).

3. Decapitate larvae, using an entomological needle. The dorsal surface of the head was pressed until the brain emerges from the posterior part of brain capsule.

4. Fix the brain tissue in Carnoy fixative (3:1 absolute ethanol : acetic acid) for 30 to 60 seconds.

5. LAO stain (2g Orcein, 50cc of 100% Lactic acid and 50cc of glacial acetic acid) is used for routine staining of chromosomes (length of time depends on intensity desired).

6. A clean cover glass should be placed on top of stain, over which gentle pressure should be applied evenly to obtain an even spread of stain.

B. SALIVARY GLAND CHROMOSOMES

1. Vigorous 4th Instar larvae were utilised for salivary gland chromosomes. (Larvae just 8 hours prior to pupation give excellent results).

2. Dissection is carried out in 5% diluted Carnoy (95 ml distilled water + 5 ml Carnoy fixative). In mosquito larvae, the bilobed salivary glands are located in antero- lateral region of thorax. A certain amount of pressure ought to be

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applied just posterior to the thorax after the head was removed and dissection needle inserted under the cuticula from the anterior.

Incision is made by rubbing a second needle over the inserted one. Interior of thorax is exposed by opening incision further and removing the gut. The overlying tissue usually covering the glands is pushed aside and both glands extricated with a needle. With practice and experience, both the glands can be removed together, if needle is placed at the junction of right and left salivary ducts.

3. After the dissection, the glands are transferred to a drop of Carnoy fixative taken on a slide. Fix gland for 1-2 minutes.

4. Stain with LAO for 5 to 10 minutes and place a cover slip. Gently apply pressure to obtain an even spread.

C. OVARIAN NURSE CELL CHROMOSOMES

1. To obtain good polytene chromosomes, ovaries of adults between 28 and 32 hours after blood meal were used.

2. Adult females ready for dissection are to be removed into a test tube and killed or stunned (by striking the lower end of the tube smartly against the palm). Each mosquito should then be placed on the slide dorsally with abdomen directed towards the right, and a small drop of dissecting fluid should be placed near it.

3. Hold anterior part of abdomen with one needle (left hand) and penultimate segment of abdomen with another and give a sharp pull posteriorly to cut the last two segments. The abdomen is gently pressed to exude the ovaries, or they emerge as a bunch.

4. Further dissection of ovaries can be done to separate the follicles for easier squashing. Fix in Carnoy fixative for 2-4 minutes.

5. Stain in LAO for 5 to 10 minutes (time may vary with amount of intensity desired).

6. Place cover slips and apply gentle pressure to obtain an even spread.

D. MEIOTIC CHROMOSOMES

1. Select out freshly pupated male pupae (small in size with respect to female pupae), pretreat with 0.01% colchicine overnight.

2. Pick pupae with eye dropper and place on clean slide, drain out moisture, add a drop of dilute Carnoy (1 part Carnoy : 19 parts distilled water). Placing the left hand needle at the thorax region, use the needle in the right hand to stretch out the pupae by placing on the third last abdominal segment. The gonads can be easily seen as light yellowish bodies on either side of the gut. Tease out the gonads gently.

3. Clean and place the gonads in hypotonic solution (Potassium Chloride, 932 mg/100ml) for 15 minutes.

4. Transfer the gonads to Carnoy fixative (3 : 1 absolute ethanol : acetic acid) for 1 to 2 minutes.

5. Stain in LAO for 10 minutes.

6. Add a drop of 45% acetic acid and place the cover slip. Seal with nail polish and observe.

E. AIR-DRYING TECHNIQUE FOR PREPARATION OF CHROMOSOMES

The air-drying technique is routinely used to obtain chromosomes spread and involves a combination of hypotonic pre-fixation treatment and air-drying. The larval and pupal tissues are used in the preparation of chromosome slides.

Prometaphase and metaphase stages are obtained from the brain, egg rafts, gonads (testes and ovaries) and their supporting tissues.

Pachytene and other meiotic stages are also obtained from gonads, while polytene chromosome nuclei are derived from the gut epithelium Malpighian tubules, salivary gland and ovarian nurse cells.

If material is scarce and there is difficulty in securing sufficient, number of metaphase plates, the larvae may be pre-treated with 0.1% Colchicine for 2-5 hours.

Methodology:

1. Material Collection

In case of mitotic chromosomes, brain tissues (approximately 10-15 larvae have to be dissected and pooled), egg rafts (approximately 300 eggs or 5 egg rafts in case of Culex are to be collected 8-12 hours after laying, these having been maintained at 20°C).

For meiotic slide preparation, male larvae are distinguished by taking into account the width of the thoracic segment; females have a wider thoracic segments than males. Two sharp incisions are made one on either side of the region of the gonads which are situated in the abdominal region. Gonadal region is segregated and transfered to cavity dish containing hypotonic solution where it is minced.

2. Hypotonic Treatment

Transfer the dissected material to cavity dish containing appropriate hypotonic solution to expand the cells.

Best preparations can be obtained with salt preparations of the following molarities

i. 0.15M Potassium Chloride, in the case of pachytene and other early meiotic stages

ii. 0.06M to 0.08M Sodium Chloride solution in case of mitotic and later - meiotic stages.

iii. 0.016M Sodium Citrate solution in the case of somatic metaphase chromosomes.

3. Processing of cell suspension

a. Dissected material in hypotonic solution should be thoroughly minced with the aid of a pair of small scissors with a curved tip until a fine cell suspension is obtained.

b. Transfer the cell suspension into a 15ml centrifuge tube using a Pasteur pipette. Centrifuge at 800 rpm for 4 minutes.

c. Discard supernatant and replace with the hypotonic solution, allow resuspension for some time and followed by centrifugation (800 rpm for 4 minutes).

d. Discard supernatant and replace with 3-5ml of freshly prepared fixative (Absolute methanol 3 parts + 1 part Glacial Acetic acid). The cell pellet should be dispersed in the fixative by gentle agitation with the help of a Pasteur pipette and volume slowly increased by addition of more fixative.

e. Centrifuge and resuspend with 2 more changes in fresh fixative. After last change add a small volume of fixative to obtain a turbid cell pellet.

4. Preparation of Slides

a. Refrigerate (i) Coplin jar with slides immersed in absolute alcohol, and (ii) Coplin jar containing distilled water some time prior to slide making.

b. Transfer chilled slides to Coplin jar containing distilled water, vigorously shake remove dirt and until smooth water surface appear.

c. Using a Pasteur pipette drop 3-4 drops of cell pellet over wet slide held at an inclined angle. Shake slide vigorously to remove excess liquid and air dry on hot plate at 40° C for 1-2 minutes.

The slides are now ready to for staining. A description of several staining protocols including. A brief description of staining protocol is given as under :

1. Aceto Orcein Staining

Slides may be stained in 1% Aceto Orcein following conventional procedure to study general morphology of mitotic and meiotic chromosomes.

2. Conventional Giemsa Staining

Slides may be immersed in dilute Giemsa Staining solution [Giemsa Staining solution is prepared by adding 1ml of Giemsa Stock solution (Harleco, original blend) and 1ml Sorenson phosphate buffer (pH at 6.8) to 48ml distilled water].

Stain slides for 5 minutes briefly, then rinse in distilled water before air drying over hot plate at 40°C.

3. G-Banding Technique (Modified method of Seabright 1971 is adopted)

Trypsin solution (0.25%) is further diluted with Hank's solution without Ca++ and Mg++.

Place slides on horizontal plane with the surface carrying cell suspension facing upwards and flood with diluted trypsin solution for 30-90 seconds before draining off. Dip slides twice in physiological saline and air dry (The air dried slides may be observed under a phase contrast microscope prior to staining).

Flood slides with dilute Giemsa solution (1ml Giemsa stock solution and Sorenson phosphate buffer with 97ml distilled water) for less than a minute and air dry.

4. Q-Banding Technique

Q-Bands are obtained with Quinacrine mustard as described by Casperson et al (1970-71).

Place destained slides in distilled water for approximately 5 minutes and stain with Quinacrine Mustard (or Hoechst 33258) solution for about 10 minutes. Staining solution is prepared by dissolving 1mg of Quinacrine mustard (or Hoechst 33258) in 20ml of distilled water (0.05mg/ml).

Place slides in McIlvaine's buffer at pH 5.5 for approximately 5 minutes and mount in buffered glycerine (McIlvaine's buffer and glycerine, 1:1) using long cover glass.

Blot out excess mounting solution with the aid of Whatman filter paper.

5. R-Banding Technique

(Technique of Lubs 1976 is routinely used for consistent and reproducible results). Incubate slides in fresh phosphate buffer [32 cc of 0.07M disodium dihydrogen

phosphate - dodeca hydrate (Na_2HPO_4 + 68 cc of 0.07M KH_2PO_4) at pH 6.5] for 20 to 25 minutes in a fresh waterbath maintained at 85°C.

Stain in 4% diluted Giemsa staining solution for 15-20 minutes.

6. C-Banding Technique

(Standard procedure for obtaining constitutive hetero chromatin).

Treat slides for 1 hour with 0.2N HCl at room temperature and then briefly rinse in distilled water.
Treat with 5% aqueous solution of barium hydroxide octahydrate at 50°C for 4-5 minutes followed by thorough washing in running tap water for few minutes.

Incubate slides for 1-4 hours in 2X SSC solution at 60°C, stain for 1-2 hours in dilute Giemsa staining solution (buffer pH at 6.8).

C-Banding technique (an alternate procedure)

This simple and rapid C-Banding technique requires no heat, alkali or acid post-fixation treatment prior to staining.

Incubate cell suspension in hypotonic sodium citrate (0.016M) solution for approximately 1 hour at room temperature.

Fix material and air dry. Slides may also be stained by conventional Giemsa banding technique to obtain deeply stained centromeric region.

7. N-Banding Technique

Incubate slides in 1M sodium phosphate (pH 4.15) for 5-10 minutes at 94±1°C.

Rinse in distilled water and stain for 30 minutes with dilute solution of 10% Giemsa stain.

Rinse briefly in distilled water and air dry.

8. Silver Staining Technique

Pre-treat slides in borate buffer solution (0.1M, pH 9.0) for 5-20 minutes at room temperature.

Stain for 24-50 hours at 50°C in Coplin jar containing 50% silver nitrate solution. Counter stain slides with dilute 2% Giemsa solution.

9. Procedures to destain the Giemsa Stained Slides.

Dip slides in xylene, air dry and place in mixture of xylene and absolute alcohol (1:1). for 10 niin, in each case.

Transfer slides to absolute alcohol for 5 minutes.

Pass the slide through different grades of alcohol consisting of 95%, 90% and 80% for 5 minutes in each case before finally passing through 70% alcohol for 60 minutes and air dry.

F. LIFE CYCLE OF MALARIAL PARASITE

There are approximately 120 parasite species belonging to genus Plasmodium which infect birds, reptiles and mammals including rodents, bats, primates and human beings. Four species of malaria parasites infect man in nature. These are

(1) *Plasmodium falciparum* (cause malignant tertian malaria)
(2) *Plasmodium vivax* (cause benign tertian malaria)
(3) *Plasmodium malariae* (cause quartan malaria)
(4) *Plasmodium ovale* (cause ovale tertian malaria)

The life cycle of all the species of human plasmodium follow a similar pattern and is completed in two hosts, a vector female *Anopheles* mosquito and a human host.

Cycle in Mosquito Host (Sporogony)

1. When a female mosquito sucks blood of an infected human host with malarial parasite in blood circulation, the asexual stages of malaria parasites are digested along with red blood cells in the gut. The sexual stages i.e. gametocytes undergo further development forming two types of gametocytes, male and female. Each male gametocyte develops further and undergoes a reduction division forming 4 to 8 male gametes which are like thin threads called flagella, each containing a nucleus and is known as microgamete. Each female gametocyte forms a single female gamete upon maturation known as macrogamete by discarding extra chromatin material by forming polar bodies.

2. In the stomach of the female, each male gamete swims actively in the blood medium and on coming in contact with a female gamete pierces through its plasma membrane, both fusing to form a zygote. Within 18 to 24 hours the zygote gets elongated with a pointed end and becomes mobile. This stage is called ookinete. The ookinete with the help of its pointed end pierces through the stomach wall of the mosquito host and comes to lie between endothelial cells of mosquito stomach. It then becomes circular in shape like a small sphere known as oocyst.

3. Oocyst gradually grows in size and appears on the outer wall of the stomach as a semi-transparent body containing grains of pigments. The nucleus divides repeatedly as it grows and finally thread-like sporozoites are formed in it. There may be as many as 200 oocyst on the gut wall of a highly infected vector mosquito.

4. The matured oocyst ruptures and releases sporozoites in the body cavity / haemocoel of the mosquito. These swim in the fluid filled haemocoel and finally lodge in the salivary gland cells. Such a female upon acquiring sporozoites in it's salivary glands becomes infective. When the infective vector female bites a healthy person the sporozoites are injected through the wound and passed into the blood stream which marks the beginning of the cycle in human host.

Cycle in Human Host

Tissue Phase

After sporozoites are injected into human host they enter into the parenchymal cells of the liver and multiply immediately (a process which is known as Pre-erythrocytic schizogony). After 6-16 days depending upon the species of malaria parasite, a schizont is formed in every infected liver cell which contains thousands of small parasites called merozoites. Finally the infected liver cells burst to release these merozoites in blood circulation.

Erythrocytic Phase

The merozoites released in the blood from the liver attach to the receptor sites on RBCs' and make an entry. It now becomes ring shaped and further grows into a disfigured amoeboid structure, This early stage of parasite in the RBC is called trophozoite which starts growing by consuming nutrients in the RBC. In the course of development of a trophozoite, the haemoglobin of the infected RBC is converted into a toxic pigment called haemozoin. This iron containing brownish pigment can be seen in the body of the parasite in a stained film.

After a period of growth, each trophozoite undergoes asexual division (a process known as erythrocytic schizogony). The nucleus of the parasite divides 3 to 5 times to produce variable number of small round or ovalish merozoites depending upon parasite species. When the process of erythrocytic schizogony is complete, the merozoites are released in the blood stream. They now seek and enter in fresh RBCs' and start the cycle all over again. Some of the merozoites, however, differentiate in to sexual stages i.e. male and female gametocytes. It is these sexual stages which infect the vector female mosquito when it acquires them along with blood of malaria patient.

G. DISSECTION AND OBSERVATION OF OOCYSTS FROM INFECTED MOSQUITO MIDGUT

1. Place the specimen on a clean slide, under a dissection microscope, remove wings and legs, and decapitate.

2. Place a drop of normal saline (0.85%) on the glass slide and place the trimmed insect close to the drop, with the abdomen pointing towards the observer.

3. Using a dissecting needle nick the chitinous skin at the sites of the abdomen between segment 6 & 7.

4. Holding the thorax with one needle place the other needle across the tip of abdomen and draw the abdominal contents out with a gentle traction, so that they touch the drop of saline.

5. When the contents, including the midgut, come out, cut the tip of the abdomen, so that ovaries, hindgut and the malpighian tubes are discarded and only midgut is retained.

6. Place gently a cover slip on to the isolated midgut and examine under a comound

microscope for the presence of oocysts. Use low power first and confirm under higher magnification. The oocyst lie on the outer surface of the midgut and appear as clear round or oval bodies, containing distinct granules. Larger more mature oocyst are 30-60 um in diamter, they have lost the pigment and are filled with hundreds of sickle shaped sporozoites, which escape from the oocyst when the latter is ruptured by a gentle pressure on the cover slip.

7. Fix the mosquito mid-gut containing oocyst with formal saline solution [1 part formalin + 9 parts normal saline] (use Pasteur pipette and dispense fixative along sides of the cover slip).

8. After fixation, remove the cover slip from the slide with distilled water using Pasteur pipette. The gut should not be allowed to dry at this stage.

9. After washing, stain the gut with Toluidine Blue stain (0.1 gm of toluidine blue + 100 ml distilled water) for one and half minute and then wash in distilled water.

10. The stained oocyst are dehydrated through Acetone Xylene mixture in the following way :

i)	95 parts Acetone + 5 Parts Xylene	-	1/2 minute
ii)	70 parts Acetone + 30 Parts Xylene	-	1/2 minute
iii)	30 parts Acetone + 70 Parts Xylene	-	1/2 minute
iv)	Pure xylene - 2 minutes and ultimately mounted in DPX.		

H. IDENTIFICATION OF MALARIAL SPOROZOITES IN MOSQUITO SALIVARY GLAND

Salivary gland dissection (Adult) (Choudhury and Ghosh, 1982)

1. Transfer mosquito to be dissected on to a glass slide (after stunning it). Remove wings and legs. Place insect in the centre of a dry slide with anterior end pointing to right, and with needle in the right hand, cut off head. Add a drop of saline to sample.

2. Holding the dissecting needle in the left hand firmly plant on the thorax just below the parts where the glands lie.

3. With needle in the right hand, bring pressure to bear on the thorax so as to express the glands from it. The glands appear as small, sausage shaped, refractile bodies, 30-50 μm in length.

4. Cover the glands with small cover slip to rupture them. Apply gentle pressure, examine under high power to observe for presence of sporozoites.

5. Fix with formal saline by running it along the sides of cover slip with the aid of Pasteur pipette.

6. Remove formal saline next by repeated changes of distilled water (4 times with Pasteur pipette)

7. Remove cover slip with needle, dry the material completely. Mark the area containing glands by glass marking pencil. Place cover slip on another slide for processing. Stain with Leishman stain diluted with distilled water at 1:4 ratio for 30 minutes, disturbing the scum every 3 or 4 minutes.

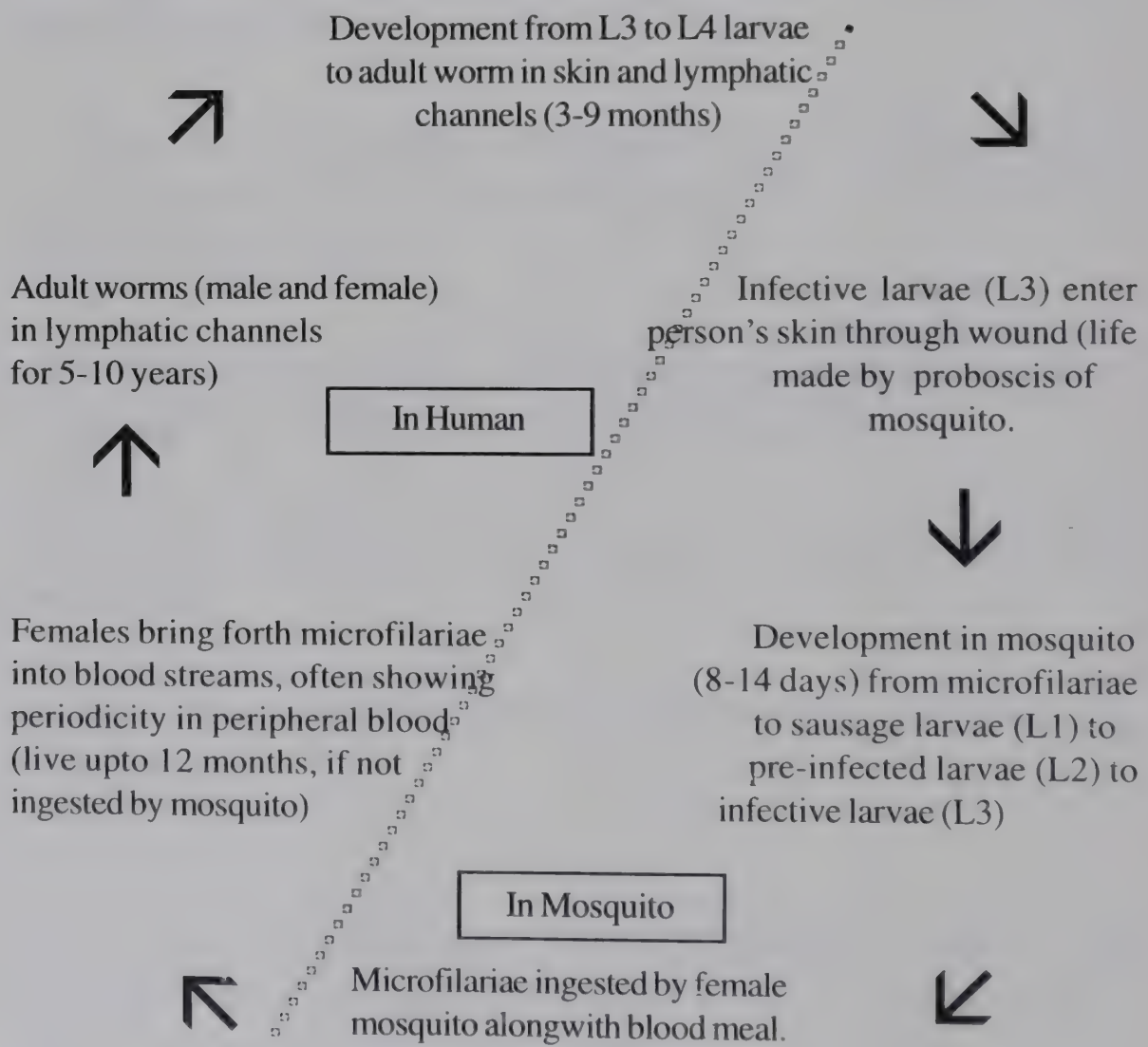
8. Wash by repeated changes of distilled water, air dry.

9. Mount with DPX. Place cover slip.

I. HUMAN LYMPHATIC FILARIASIS

Human lymphatic filariasis results from infection with the nematode parasites Wuchereria bancrofti, and Brugia malayi. The juvenile and adult worms normally live in the lymph vessels and lymph nodes and the microfilariae are found in blood. The adult parasites can live for many years (probably upto 10 years, but a 40-year life-span has been reported). The life span of microfilariae is about a year at the most.

Life cycle of parasite


Development from L3 to L4 larvae to adult worm in skin and lymphatic channels (3-9 months)
Adult worms (male and female) in lymphatic channels for 5-10 years)
Infective larvae (L3) enter person's skin through wound (life made by proboscis of mosquito.
In Human
Females bring forth microfilariae into blood streams, often showing periodicity in peripheral blood (live upto 12 months, if not ingested by mosquito)
Development in mosquito (8-14 days) from microfilariae to sausage larvae (L1) to pre-infected larvae (L2) to infective larvae (L3)
In Mosquito
Microfilariae ingested by female mosquito alongwith blood meal.

Dissection of mosquitoes for microfilariae

1. The parasite is transmitted by mosquitoes which serve as intermediate host in which microfilariae develop to the infective stage.

2. The slender active microfilariae transforms to the short thick inactive sausage-stage or L1 larvae (The L1 larvae has a cuticle which forms a conspicuous slender tail, characteristic of the stage).

3. After the first moult, the larvae grow rapidly in length and width and becomes potentially more active, although usually it does not move. This L2 or pre-infective larvae is recognised by its short tail and presence of one or two papillae at the caudal end.

4. After second moult the parasite no longer has a visible cuticle and is called the infective or L3 larvae.

5. The L3 larvae moves actively in the haemocoelic cavity of mosquito first towards abdomen and later to the head and proboscis (where most of them are found upon dissection).

6. When the infective mosquito takes a blood meal, some or all of the infective larvae escape from proboscis and actively enter human host.

7. The L3 larvae develops in the lymphatic system to L4 stage, to the young adult stage and finally to the mature adult worm, male or female.

8. After fertilisation, the female worms produce microfilariae which find their way from lymphatic systems to the blood stream.

Figure 1 : Photomap of Polytene Chromosomes of *'Anopheles stephensi'* from Ovarian Nurse Cells

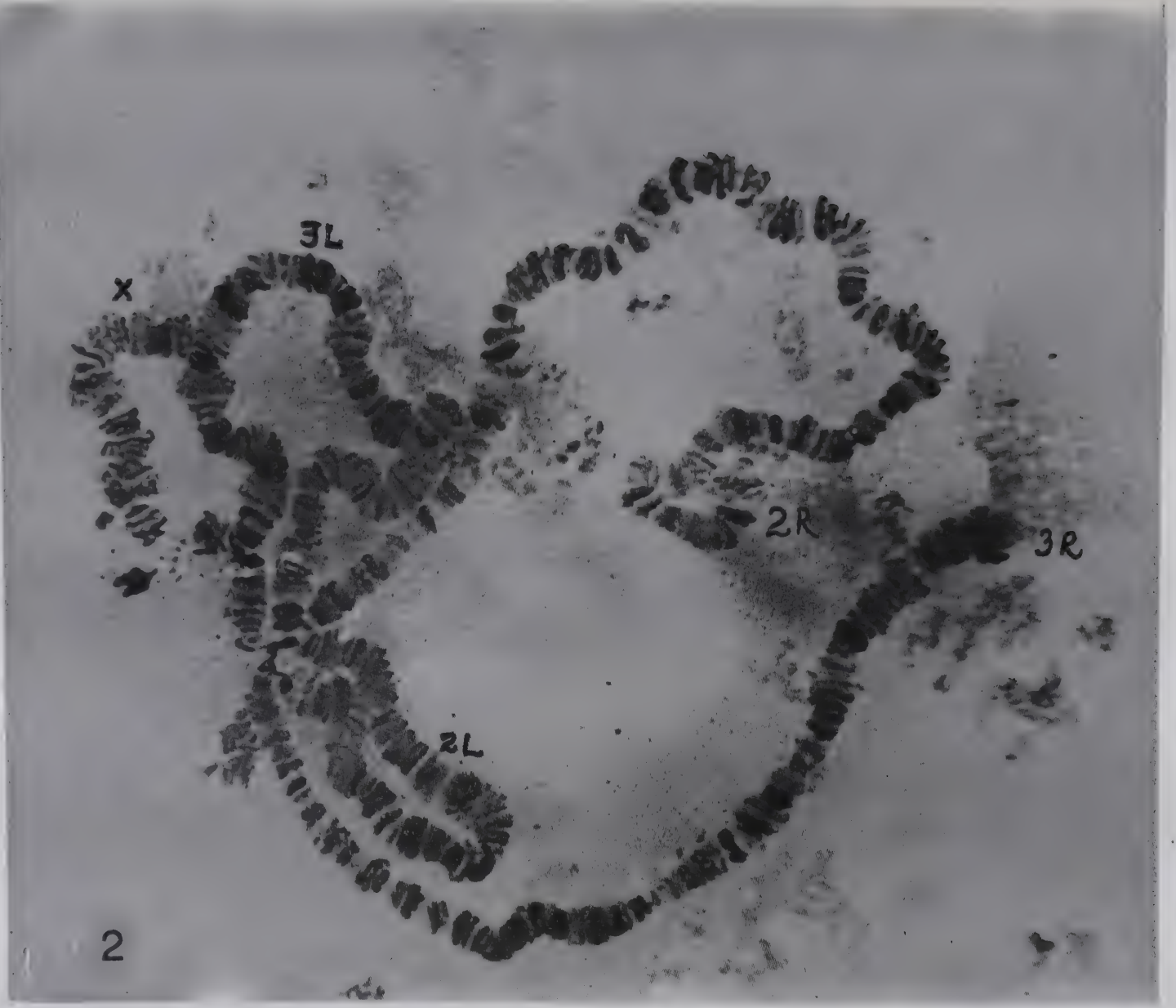


Figure 2 : Polytene Chromosome Complement of *'Anopheles culicifacies'*

Figure 3 : Inversion in Arm 2L (f / +) in *Anopheles stephensi* (Ovarian Nurse Cell preparations

Figure 4 : Inversion in Arm 2R (b / +) in *Anopheles stephensi* (Ovarian Nurse Cell Preparation)

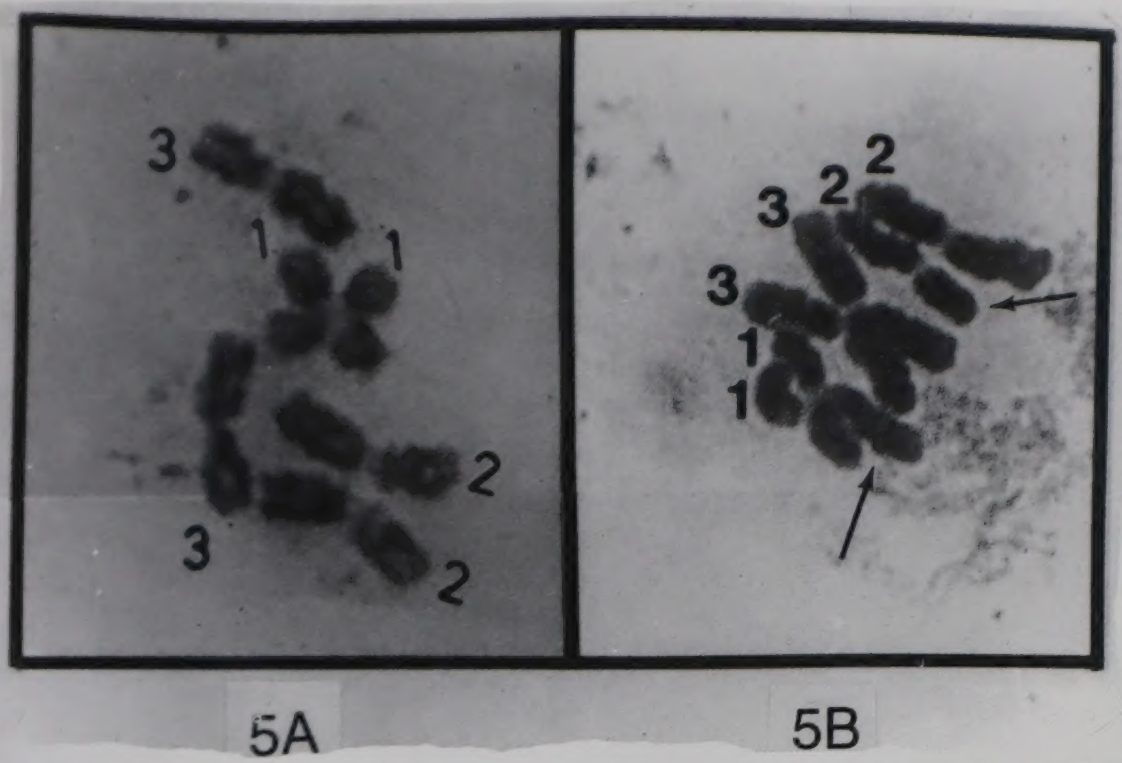


Figure 5A : Normal Mitotic Metaphase Chromosome from Pupal Testis of *Anopheles stephensi*

Figure 5B : Metaphase Chromosomes from Pupal Testis Showing Translocations (1:2) *(Anopheles stephensi)*

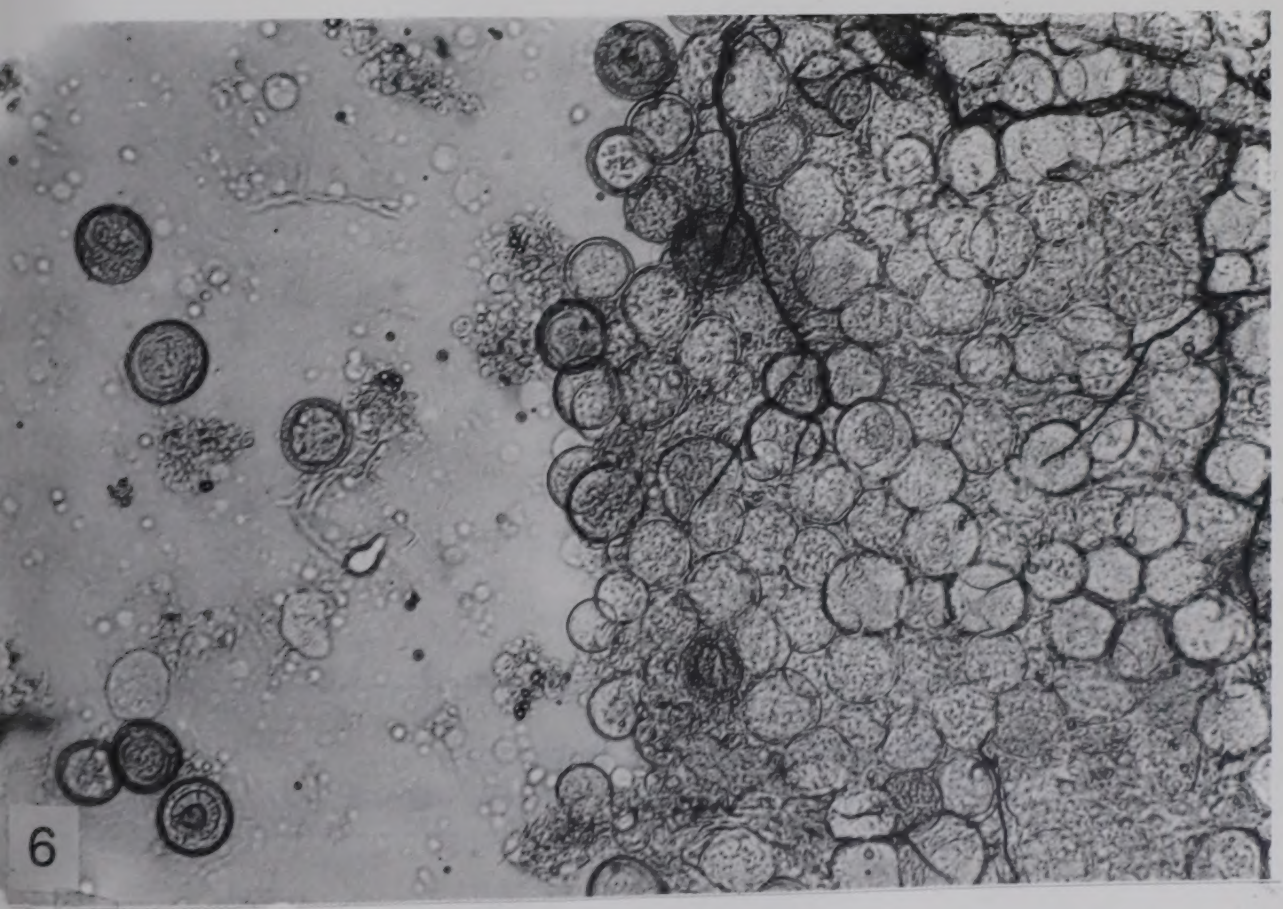


Figure 6 : Oocyst of *Plasmoduim* in the Gut of Anopheles *quadrimaculatus*

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